

AWARD NUMBER: W81XWH-14-1-0543

TITLE: An in Vivo Investigation of Brain Inflammation in Gulf War Illness with Integrated PET/MR Imaging

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2014 - 29 Sep 2015	
4. TITLE AND SUBTITLE An in Vivo Investigation of Brain Inflammation in Gulf War Illness with Integrated PET/MR Imaging				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-14-1-0543	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Marco Loggia, PhD E-Mail: marco@nmr.mgh.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) MASSACHUSETTS GENERAL HOSPITAL, THE SUSAN ROUDEBUSH 55 FRUIT ST BOSTON MA 02114-2621				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This project is aimed at evaluating the contribution of brain glia to the pathophysiology of Gulf War Illness, as well as fibromyalgia (a functional pain disorder characterized by similar symptoms). So far we have completed administrative and regulatory review, and have begun subject enrollment. The first two participants have completed the study. An additional three participants were enrolled and found to be ineligible at the time of screening. Throughout the recruitment process, we have actively modified our inclusion criteria to address concerns of ambiguity as they have arisen. Recruitment has been slower than anticipated, but efforts are well underway to improve recruitment procedures. Additionally, we experienced recent delays as a result of the Martinos Center cyclotron undergoing repair for more than one month. However, the resolution of these hurdles is in sight, and we are confident that we'll soon be able to increase our recruitment rate and successfully complete the project within the expected timeframe.					
15. SUBJECT TERMS Recruitment					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:

In this project, we are using simultaneous magnetic resonance imaging (MR) and positron emission tomography (PET) with [^{11}C]PBR28 – a recently developed PET ligand which binds to activated microglia with unprecedented specific-to-nonspecific binding ratio – to test the hypothesis that patients with Fibromyalgia (FM) or Gulf War Illness (GWI) demonstrate over-activation of brain microglia. Microglia are a subpopulation of macrophages known to mediate the inflammatory response of the central nervous system. While under normal conditions these cells are involved in adaptive homeostatic defense responses, such as the destruction of invading microorganisms, animal models have also provided evidence for a role of microglial activation in the development of chronic pain. Recognizing the role of these chronically active microglial cells in FM and GWI might lead to the development of new and potentially more effective treatment approaches for both conditions. Furthermore, if disease-specific patterns of microglial activation can be identified, it would improve our ability to correctly diagnose the two conditions and treat them with specificity not possible to date.

2. KEYWORDS:

Fibromyalgia; Gulf War Illness; Chronic Pain; Microglia; Neuroinflammation; Positron Emission Tomography; Magnetic Resonance Imaging

3. ACCOMPLISHMENTS:

What were the major goals of the project?

As stated in approved Statement of Work, we anticipated the following accomplishments during Year 1 of the project:

- Task 1: Regulatory Review and Approval Processes (Partners IRB and DoD HRPO; months 1-3)
- Task 2: Subject Recruitment (months 4-24)
- Task 3: Study Visits (Behavioral and Imaging; months 5-32)
- Task 4: Data Analysis (Data Preprocessing; months 5-32)

Based on our approved quarterly enrollment targets, we anticipated enrolling and scanning 13 participants in Year 1.

What was accomplished under these goals?

Of the above stated goals, we have successfully completed Task 1 and are making steady progress in Tasks 2-4. The Regulatory Review and Approval Processes (**Task 1**) began immediately upon receipt of the award from the Department of Defense, in October 2014, but final protocol approval from both the Partners IRB and the DoD HRPO was not received until April 24, 2015. Due to the necessity of a complete protocol review by both our institutional IRB and the DoD HRPO, as well as a subsequent re-review for any requested changes by either review board, the approval process was longer than anticipated.

Subject Recruitment (**Task 2**) began immediately after protocol activation was received in April 2015. As outlined in our initial application, we anticipate that the majority of study participants (Gulf War veterans, with and without Gulf War Illness) will be recruited from among the 175 participants enrolled in the Gulf War Illness Consortium being conducted at the Boston University School of Public Health, and co-Pled by Drs. Sullivan and Krengel (respectively co-Investigator and consultant to the present project). Unfortunately, that study has experienced some delays in the recruitment procedures, which has limited our access to veterans. However, the Consortium's recruitment rate is picking up, and we are therefore still confident that we will be able to recruit 16 Gulf War Illness veterans and 16 healthy veteran controls within the expected timeframe.

While we wait for the recruitment of veterans to pick up, we have started focusing on Fibromyalgia (FM)

patients. Fibromyalgia patients are being recruited directly by our own study staff from rheumatology and pain management clinics in the greater Boston area, from flyers and advertisements posted within the Partners Healthcare campuses, and through online resources offered by Partners Healthcare, such as ‘RSVP for Health.’ Our early efforts have made extensive use of ‘RSVP for Health’ – the Partners Healthcare-based tool for study recruitment. Of the forty-eight potential FM participants who completed pre-screening, only five have been enrolled, and only two have been eligible for study completion. Given this low yield, we have recently decided to adjust our recruitment strategies (see “**What do you plan to do during the next reporting period to accomplish the goals?**”), and have immediately seen promising increases in contacts by patients. To supplement recruitment, we have also taken advantage of contact lists of Fibromyalgia patients from our previous functional MRI studies. Of the 33 participants in our records who meet age- and genotype-related inclusion criteria, 11 were successfully contacted and discussed study participation.

Figure 1 below outlines the exclusion criteria met by all 57 individuals screened to date for study participation.

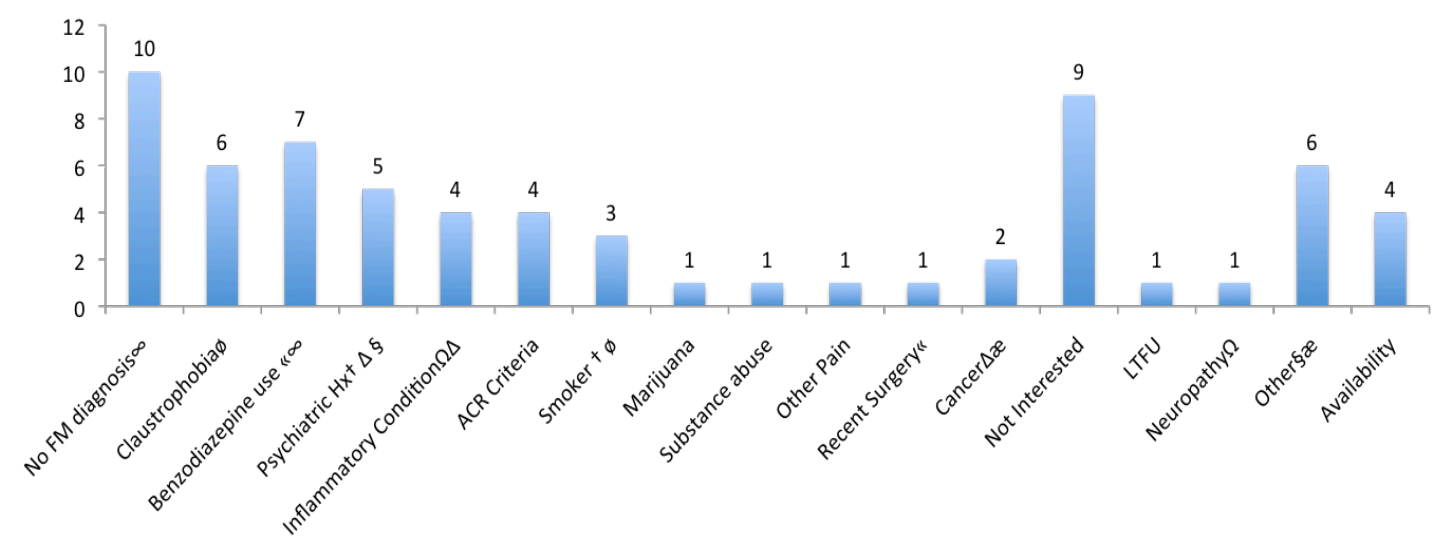


Figure 1: Exclusion Criteria Satisfied by Fibromyalgia Patients Pre-Screened for Study Participation. A total of 57 potential participants diagnosed with Fibromyalgia completed an over-the-phone prescreen. Nine symbols (†ø«ΔΩ∞§æ) indicate individuals excluded in multiple categories. LTFU – Lost to Follow-Up.

In terms of Study Visits (**Task 3**), so far five participants have completed behavioral visits, and two completed also the imaging visit (Figure 2). The remaining participants were withdrawn for significant history of psychological illness (2) and comorbid autoimmune disorder (1). An additional two participants were found eligible, but are currently unavailable for participation.

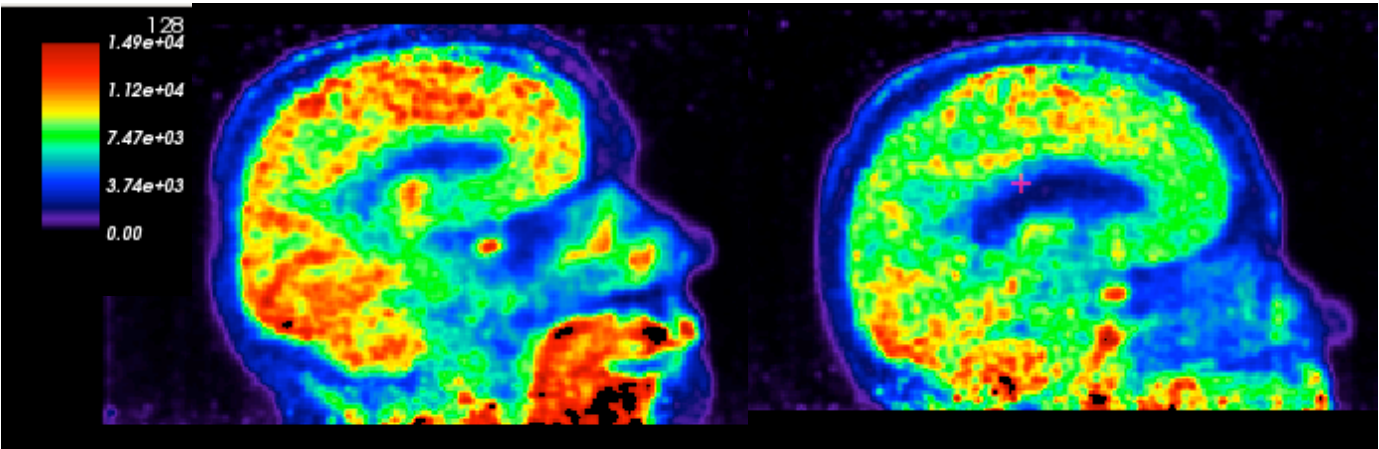


Figure 2: Mean 0-90min [^{11}C]PBR28 PET images from our first two fibromyalgia patients scanned (color scale represents tissue radioactivity, expressed in bq/ml)

In January of 2014, our recent findings on microglial activation in chronic low back pain were published in *Brain*, in a paper titled 'Evidence for brain glial activation in chronic pain patients' (Appendix 1). The imaging methods – including the identical use of [^{11}C]PBR28 as a marker of glial activation – mirror those of the present study and provide the first evidence that glial activation can be observed in patients suffering from a chronic pain disorder. The success of the previous study has also been a significant contributing factor to some of the modifications made to the present protocol (see Section 5, arterial line removal).

What opportunities for training and professional development has the project provided?

Thanks to the Award, the PI had the opportunity to present at national and international conferences his work on glial imaging: the Society for Neuroscience (SfN) meeting in Washington, DC and the meeting of the Neuropathic Pain Special Interest Group (NeuPSIG), in Nice. In the latter, he chaired a workshop on the role of glial in pain.

How were the results disseminated to communities of interest?

The findings from this recent *Brain* paper have been the subject of a number of talks by the PI in this past year, including locally (e.g. a seminar at VA Boston Healthcare System, a meeting of the Gulf War Illness Consortium, held at Boston University), and at national and international conferences (NeuPSIG in France and SfN, in Washington, DC). However, this particular project has not yet yielded significant results to share.

What do you plan to do during the next reporting period to accomplish the goals?

Our goals and objectives will remain largely unchanged through the next reporting period. We will continue with efforts to accomplish Tasks 2-4, as outlined in the SOW. We are actively pursuing other means of study recruitment, including the Research Patient Data Registry (RPDR) – a Partners Healthcare-based tool designed to provide patient information to researchers. We have also begun efforts to build direct relationships with rheumatologists in the Partners Healthcare system in order to recruit interested patients directly from the clinic. This strategy has been working very well, and within 1 week we have received six patient referrals to the study. Of these, one patient is scheduled for on-site screening and enrollment, and another is pending pre-screening for study eligibility. We are confident that this strategy will soon allow us to recruit the target number of participants.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

All of the changes described below were reported to the DoD HRPO at the time of their approval by the Partners IRB. None of the changes listed required full meeting review by either the Partners IRB or the DoD HRPO. All changes have also been reported in the quarterly technical progress reports.

Changes in approach and reasons for change

1. Removal of arterial line during brain imaging: Typically, arterial lines are necessary in order to perform tracer kinetic modeling. However, for [11C]PBR28 – the radiotracer used in this study – our group and others (Lyoo et al., J Nucl Med, in press) have found that a simple ratio method (SUVR; standardized uptake values ratio), which does not require the sampling of arterial blood, can substitute for and may even be more sensitive than absolute quantification through kinetic modeling. The removal of the arterial line also significantly reduces the risks and discomforts participants may experience during the course of their participation.

2. Addition of 16 healthy civilian controls: This amendment will allow us to better account for any residual demographic differences between Fibromyalgia and Gulf War Illness patients. While we are placing particular efforts into the recruitment of gender-balanced samples, and while the recently proposed American College of Rheumatology criteria for fibromyalgia identify more males than in the past, we nonetheless recognize that there will likely be remaining gender differences in our final sample. We have, therefore, decided to add an additional control group consisting of 16 healthy civilian volunteers to match the fibromyalgia patients. This additional control group will also allow us to investigate any differences that may result from combat exposure. As outlined in the June 2015 technical report, Department of Defense funds will not be used to cover the costs associated with these 16 additional participants. They are to be paid for using funds from an R21 grant from the NIH-NINDS.

3. Minor modifications to inclusion/exclusion criteria: Minor changes have been made to the study criteria in order to eliminate ambiguity. The need for these amendments became apparent during the recruitment process, as potential participants with medical histories not directly addressed by the original criteria inquired about the study. Additional minor modifications were made to ensure the quality of brain imaging data and the blood cytokine panel (e.g., exclusion for active bacterial or viral infection, use of anti-inflammatory medications). These changes are further outlined in the technical reports.

Actual or anticipated problems or delays and actions or plans to resolve them

We have had few problems or delays to date. As detailed in Section 3, the initial protocol activation process took several months longer than anticipated, due in part to our inexperience with the HRPO's regulatory reporting process. Nonetheless, the protocol was successfully approved and we now find ourselves well familiarized with and able to navigate the reporting requirements.

We have also faced some difficulties with the recruitment of patients. As we wait for recruitment to increase at the Boston University Gulf War Consortium (which is currently ramping up), we have focused our own recruitment efforts on Fibromyalgia patients.

Again, as detailed in Section 3, we have relied heavily on RSVP, a study recruitment tool provided by Partners Healthcare. While RSVP for Health has in the past proven an excellent recruitment tool for more commonplace conditions, such as low back pain, it has major limitations in targeting patients for a condition as complex as fibromyalgia (e.g., comorbidity, difficulty of diagnosis, medication use). The tool offers a limited number of fairly broad categories under which interested patients may register, such as 'Pain,' and does not

offer a very effective means of confirming patient diagnoses. We have found the yield from RSVP to be disappointingly low. As a result, we are actively pursuing other sources of patients, including RPDR – another Partners recruitment tool – and direct relationships with interested clinicians inside the Partners Healthcare system. Our current relationship with rheumatologists at the Massachusetts General Hospital has proven fruitful and we will continue building similar relationships with other physicians.

Finally, we had issues with the cyclotron (which is needed for the PET scanning) needing repair for more than a month. We are told that, as things now stand, it should be back up sometimes in early November. Thus, we will soon be able to resume screening and scanning.

Changes that had a significant impact on expenditures

Given that recruitment has experienced a delay, we have not yet use any budget devoted for imaging. Please note that for the two initial participants completing the study, the costs associated with scanning were paid for using an alternative source of funding – a short-term teaching grant awarded by the Martinos Center for Biomedical Imaging itself.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Nothing to report

Website(s) or other Internet site(s)

scholar.harvard.edu/loggia

This is the lab's official website, hosted within the Harvard University network. The website provides an introduction to the lab's research, recent news, and a complete list of publications.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Marco Loggia, PhD
Project Role:	Principal Investigator
Researcher Identifier (ORCID ID):	0000-0002-8026-5265
Nearest Person Month Worked:	4
Contribution to Project:	Directed the project, hired and supervised personnel, collected data
Funding Support:	

Name:	Ekaterina Protsenko
Project Role:	Research Assistant
Researcher Identifier (ORCID ID):	N/A
Nearest Person Month Worked:	3
Contribution to Project:	Recruited and screened participants, collected data
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The only investigator devoting at least 1 cal. mo. was the PI, Marco Loggia. Below are the changes in other support occurring after the beginning of the project.

New sources of support

1R21NS087472 (PI: Loggia), The Role of Neuroimmune Activation in Chronic Pain and Negative Affect (2.79 cal; previously pending)

R01 AT007550 (PI: Harris/Napadow) Neuroimaging Approaches to Deconstructing Acupuncture for Chronic Pain (1.4 cal).

Reduced effort

1R21NS082548-01A1 (PI: Zhang/Hooker), PET/MRI Imaging of Neuroaxial Inflammation in Sciatica Patients (from 2.4 cal to 0.34 cal)

NCMIC Foundation (PI: Loggia), Neural Correlates of Spinal Manipulative Therapy (from 1.92 cal to 0.6 cal)

Terminated

IASP Early Career Award (PI: Loggia), An in-vivo investigation of brain inflammation in fibromyalgia with integrated PET/MR imaging.

1R01AG03498-01 (PI: Edwards), Biobehavioral Risk Factors for Persistent Pain following Total Knee Arthroplasty.

What other organizations were involved as partners?

Organization Name:	Brigham and Women's Hospital
Location of Organization:	Boston, MA
Partner's Contribution to the Project: Financial Support In-kind Support Facilities Collaboration Personnel Exchange Other	Dr. Lee has been working with project staff on the project.

Organization Name:	Boston University School of Public Health – Gulf War Illness Consortium
Location of Organization:	Boston, MA
Partner's Contribution to the Project: Financial Support In-kind Support Facilities Collaboration Personnel Exchange Other	Dr. Sullivan has been working with project staff on the project

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report

QUAD CHARTS:

Nothing to report

9. APPENDICES:

- A. Loggia, M.L., et al., 2015. Evidence for brain glial activation in chronic pain patients. *Brain*, 138(Pt 3), pp.604-15.

Evidence for brain glial activation in chronic pain patients

Marco L. Loggia,^{1,2,*} Daniel B. Chonde,¹ Oluwaseun Akeju,³ Grae Arabasz,¹ Ciprian Catana,¹ Robert R. Edwards,^{2,4} Elena Hill,⁵ Shirley Hsu,¹ David Izquierdo-Garcia,¹ Ru-Rong Ji,^{2,6} Misha Riley,¹ Ajay D. Wasan,^{2,4,7} Nicole R. Zürcher,¹ Daniel S. Albrecht,¹ Mark G. Vangel,¹ Bruce R. Rosen,^{1,8} Vitaly Napadow^{1,2,9} and Jacob M. Hooker¹

Although substantial evidence has established that microglia and astrocytes play a key role in the establishment and maintenance of persistent pain in animal models, the role of glial cells in human pain disorders remains unknown. Here, using the novel technology of integrated positron emission tomography-magnetic resonance imaging and the recently developed radioligand ¹¹C-PBR28, we show increased brain levels of the translocator protein (TSPO), a marker of glial activation, in patients with chronic low back pain. As the Ala147Thr polymorphism in the *TSPO* gene affects binding affinity for ¹¹C-PBR28, nine patient-control pairs were identified from a larger sample of subjects screened and genotyped, and compared in a matched-pairs design, in which each patient was matched to a TSPO polymorphism-, age- and sex-matched control subject (seven Ala/Ala and two Ala/Thr, five males and four females in each group; median age difference: 1 year; age range: 29–63 for patients and 28–65 for controls). Standardized uptake values normalized to whole brain were significantly higher in patients than controls in multiple brain regions, including thalamus and the putative somatosensory representations of the lumbar spine and leg. The thalamic levels of TSPO were negatively correlated with clinical pain and levels of circulating proinflammatory cytokines, suggesting that TSPO expression exerts pain-protective/anti-inflammatory effects in humans, as predicted by animal studies. Given the putative role of activated glia in the establishment and or maintenance of persistent pain, the present findings offer clinical implications that may serve to guide future studies of the pathophysiology and management of a variety of persistent pain conditions.

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Received August 27, 2014. Revised November 12, 2014. Accepted November 19, 2014.

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Keywords: chronic pain; ^{11}C -PBR28; translocator protein (18kDa); TSPO; neuroinflammation; glia

Abbreviations: LBP = low back pain; SUV = standardized uptake value

Introduction

Until recently, chronic pain has been thought to arise primarily from neuronal dysfunction within nociceptive pathways of the nervous system (Hains and Waxman, 2006). In the last decade, however, a paradigm shift has occurred in the field of pain neurobiology. Animal studies have clearly demonstrated that microglia and astrocytes in the CNS, as well as neuro-glial interactions, play a key role in the establishment and maintenance of persistent pain (Tsuda et al., 2003; Watkins et al., 2007; Calvo et al., 2012; Ji et al., 2013). Both microglia and astrocytes respond to pathological events in CNS, such as strokes, trauma or neurodegenerative diseases, by undergoing a series of cellular responses collectively known as 'glial activation' (Gehrmann et al., 1995; Pekny et al., 2014). This response includes proliferation, morphological changes, increased or *de novo* expression of cell surface markers or receptors, and the production of cytokines and other inflammatory mediators. Normally, glial activation is an adaptive defensive mechanism that can contribute to handling acute stress, limiting tissue damage, and restoring homeostasis. However, when malfunctioning (and, in particular when it does not get resolved during the post-acute or early chronic stage after an injury event) (Rolls et al., 2009) glial activation can have deleterious effects, and turn into the primary pathogenic element (Pekny and Pekna, 2014). Several animal studies have now established that glial activation is a key contributing factor in persistent pain. It is well demonstrated that activated microglia and astrocytes can produce cytokines such as tumour necrosis factor alpha (TNFA) and interleukin 1 beta (IL1B), and these cytokines are thought to play an essential role in the pathogenesis of chronic pain (Watkins et al., 2007; Uceyler and Sommer, 2012). It has also been shown that TNFA and IL1B can directly modulate spinal cord synaptic transmission to induce central sensitization and enhance pain states (Kawasaki et al., 2008). Moreover, the intraspinal injection of activated glia produces tactile allodynia, a hallmark of neuropathic pain, in naive rats (Tsuda et al., 2003). Conversely, the injection of drugs inhibiting glial activation can inhibit, delay or reverse pain (Meller et al., 1994; Watkins et al., 1997; Guo et al., 2007; Okada-Ogawa et al., 2009). These observations, alongside other mounting evidence from laboratory models, suggest that chronic pain may result from gliopathy (Ji et al., 2013).

In humans, some observations suggest that activated glia may contribute to the pathophysiology of chronic pain. For instance, post-mortem immunohistochemical studies in the spinal cord of patients with complex regional pain syndrome (Del Valle et al., 2009) and HIV-related neuropathic pain (Shi et al., 2012), as well as CSF sampling in patients

with fibromyalgia and chronic low back pain (LBP) (Brisby et al., 1999; Kadetoff et al., 2012), support a role for glia in chronic pain. Despite these observations, however, no study has yet demonstrated *in vivo* glial activation in humans suffering from chronic pain. Clinically, acknowledging the role of glial activation in pain disorders holds significant promise for the improved diagnostic accuracy of pain disorders. It may also provide a biological basis for the assessment of existing treatments, and the development of novel ones.

In this study we tested the hypothesis that patients with chronic pain demonstrate *in vivo* activation of brain glia. To assess this hypothesis, we imaged the brain of individuals diagnosed with chronic LBP as well as pain-free healthy volunteers using a Siemens 3 T integrated positron emission tomography/magnetic resonance imaging (PET/MRI) scanner and the recently developed PET radioligand, ^{11}C -PBR28 (Brown et al., 2007; Briard et al., 2008). ^{11}C -PBR28 binds to the translocator protein (18kDa) (TSPO), a protein upregulated in activated microglia and reactive astrocytes in animal models of pain (Hernstadt et al., 2009; Wei et al., 2013), and a putative imaging biomarker of inflammation (Cagnin et al., 2007). In addition, as evidence suggests that proinflammatory cytokines are secreted by glial cells in various animal models of pain (Ji et al., 2013), we assessed blood levels of interleukin 6 (IL6), IL1B and TNFA, and evaluated their association with our imaging findings.

Materials and methods

Study design

The study was conducted at the Athinoula A. Martinos Center for Biomedical Imaging at Massachusetts General Hospital. The protocol was approved by the Institutional Review Board and the Radioactive Drug Research Committee.

Subjects

Nineteen patients diagnosed with chronic LBP for at least 2 years (either with or without radicular pain complaints) and 25 healthy controls with no history of chronic pain were initially screened to participate in the study. Individuals were excluded if they had any PET/MRI contraindications (including pregnancy, metallic implants, claustrophobia), had a history of major medical disorders, or were on benzodiazepines or blood thinners (see Supplementary Table 1 for pain history and demographic information).

The Ala147Thr polymorphism in the TSPO gene predicts binding affinity for ^{11}C -PBR28, with the Ala/Ala, Ala/Thr and Thr/Thr genotypes being associated with high, mixed and low affinity binding, respectively (Owen et al., 2012; Kreisl et al., 2013; Yoder et al., 2013). Thus, all participants

were tested for this polymorphism and individuals with predicted low-binding affinity (Thr/Thr) were excluded. Given the effect of the TSPO polymorphism on binding affinity for ^{11}C -PBR28, and the fact that the effects of sex and age on ligand binding are unknown, a matched-pairs design was adopted, in which each patient was matched to a TSPO polymorphism-, age- and sex-matched control. Of the 44 subjects initially screened, nine chronic LBP/control matching pairs (with two patients matching the same control) were identified among the initial pool, consisting of seven Ala/Ala and two Ala/Thr pairs, and five male and four female pairs, with a median age difference of 1 year (Supplementary Table 1).

Screening visit

All participants considered potentially eligible after an initial phone screening were recruited to participate in a 2-h characterization and training visit. In this visit, venous blood was drawn in order to have all participants genotyped for the Ala147Thr TSPO polymorphism, and a urine test was performed to ensure that none of the subjects were on benzodiazepines, or taking illegal drugs. At the end of the visit, all participants completed the Beck Depression Inventory (Turner and Romano, 1984; Geisser *et al.*, 1997) and the Hospital Anxiety and Depression Scale (Zigmond and Snaith, 1983). In addition, the patients completed the McGill Pain Questionnaire (MPQ), short form (Melzack, 1987).

Imaging visit

On a separate date, eligible participants were invited to participate in an imaging visit. At the beginning of the visit, venous blood was drawn to assess levels of circulating proinflammatory cytokines: IL6, IL1B and TNFA (Supplementary Table 2). These specific proinflammatory cytokines were assessed because they are secreted by glial cells in various animal models of pain (Ji *et al.*, 2013).

Brain imaging was performed with a Siemens PET/MRI scanner (Catana *et al.*, 2008) consisting of a dedicated brain avalanche photodiode-based PET scanner operating in the bore of a 3 T whole-body magnetic resonance scanner equipped with an 8-channel head coil. The use of integrated PET/MRI allowed us to collect structural MRI simultaneously with the PET data. Patients were scanned with ^{11}C -PBR28, a recently developed TSPO radioligand that displays an 80-fold higher *in vivo* specific binding than the earlier generation TSPO radioligand ^{11}C -(R)-PK11195 (Kreisl *et al.*, 2010). ^{11}C -PBR28 was produced in-house using a procedure modified from the literature (Imaizumi *et al.*, 2007).

At the beginning of the imaging visit, we performed a series of magnetic resonance scans, including a multi-echo MPRAGE volume (repetition time/echo time 1/ echo time 2/ echo time 3/ echo time 4 = 2530/1.64/3.5/5.36/7.22 ms, flip angle = 7°, voxel size = 1 mm isotropic) for the purpose of anatomical localization, spatial normalization of the imaging data, as well as generation of attenuation correction maps (Izquierdo-Garcia *et al.*, 2014). The radioligand was then injected as an intravenous bolus, with a median administered dose (interquartile range) of 11.2 (0.3) mCi for patients with chronic LBP and 11.2 (0.6) mCi for controls, and a median specific activity at time of injection of 2.7 (0.8) mCi/nmol for chronic LBP and 2.0 (1.2) mCi/nmol for controls (both values not significantly different across groups). PET data were acquired over the course of 90 min for all but two subjects (see below) and

stored in list-mode format. During the imaging visit, all subjects except one rated their level of pain using a verbal 0–100 numerical ratings scale (pain: 0 = 'no pain', 100 = 'most intense pain tolerable'). The remaining subject (a healthy volunteer) was scanned under a separate protocol, which did not call for ratings of pain before and after the functional runs (but was otherwise identical in the procedures and parameters of the imaging visit).

Data analysis

Using in-house software, standardized uptake values (SUV; i.e. mean radioactivity/injected dose/weight) were computed for each subject. SUVs have been previously used as a measure of TSPO expression in both humans (Hirvonen *et al.*, 2012; Fujita *et al.*, 2013) and rodents (Shao *et al.*, 2013). SUVs were computed voxel-wise from the 60–90 min post-injection PET data, except for two subjects (one patient and one control) for whom SUVs were computed from 72–89 min and 60–86 min, respectively, because of unavailability of the full 60–90 min frame. The two subjects matching these subjects with incomplete data were reconstructed with identical frame onset and duration for the group analyses, to ensure that differences in the PET framing scheme would not affect our results. To maximize accuracy in the reconstruction, SUVs were generated in a two-step iterative process. First, a preliminary SUV image was created for each subject using an attenuation correction map (mu-map) computed from the MPRAGE in its native space (Izquierdo-Garcia *et al.*, 2014). To account for motion that may have occurred between the time the MPRAGE and the 60–90 min PET data were acquired, a new mu-map was created from the MPRAGE registered to this temporary SUV map. This registration was performed using spmregister, a tool from the FreeSurfer suite (<http://surfer.nmr.mgh.harvard.edu>) (Dale *et al.*, 1999) that performs a rigid-body registration of a functional volume to its relative FreeSurfer anatomical volume with Normalised Mutual Information using spm_coreg from the SPM suite (<http://www.fil.ion.ucl.ac.uk/spm/>) (Friston, 2003). A final SUV map was created using this new mu-map, now well in register with the 60–90 min PET data. SUV maps were then normalized to MNI space using non-linear registration (FNIRT, from the FSL suite; FMRIB's Software Library, version 4.1.9, www.fmrib.ox.ac.uk/fsl/) (Smith *et al.*, 2004). Spatially-normalized SUV images were then spatially smoothed (full-width at half-maximum = 8 mm) to improve signal-to-noise ratio, and intensity-normalized to a mean of 1 (SUVs) in order to account for global signal differences across subjects, such as those introduced by differences in the Ala147Thr polymorphism, which affects global binding affinity.

Although the subjects scanned were carefully selected from a larger sample size to match for genetic and demographic factors, the size of our final sample was relatively small. For this reason, imaging and behavioural group comparisons were performed using non-parametric testing. As glial activation has been postulated to spread transynaptically from the site of an injury, following the lesioned neural pathways (Banati, 2003), we hypothesized that in patients with chronic LBP glial activation would be detected in early pain processing regions, as they are most proximal to the pathological sites in the back (e.g. spinal cord/nerve roots). Thus, a thalamus-targeted region of interest analysis was first performed to test this regionally-specific

hypothesis. In this analysis, a matched-pairs test (sign test) was performed on the mean SUVRs extracted from the voxels within the right and left thalamus labels of the Harvard-Oxford Subcortical Structural Atlas (Centre for Morphometric Analyses, http://www.cma.mgh.harvard.edu/fsl_atlas.html), thresholded at the arbitrary value of 30.

We then performed a whole-brain, voxel-wise, matched-pairs analysis with the purpose of identifying (i) which thalamic subregion would be driving the effect in the region of interest analyses (if any was detected); and (ii) additional regions of glial activation within the entire brain. This analysis was conducted using the non-parametric randomize tool from the FSL suite (Nichols and Holmes, 2002), with 10 000 permutations and 5 mm variance smoothing (which increases power with smaller sample sizes: <http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Randomise/UserGuide>). Finally, as exposure to opioids may also lead to a glial reaction *per se* (Watkins et al., 2009), we performed the same SUVR group analysis after the exclusion of the data from the two patients on opioids (and their matching controls; seven versus seven), and limited our search to significant clusters from the original analysis. In all cases, whole-brain voxel-wise group comparisons maps were thresholded using threshold-free cluster enhancement (Smith and Nichols, 2009), using a corrected threshold of $P = 0.05$. For both region of interest and whole-brain voxel-wise analyses, the comparison of nine patient-control pairs was repeated (for consistency sake) in two separate matched-pairs analyses, each using one of the two patients matched to the same control. In the voxel-wise analyses, we first performed a whole-brain search using the data from the patient that best matched the control in terms of age; subsequently the voxel-wise analyses were repeated using the data from the other patient, limiting our search to significant clusters from the first analysis.

Group comparison of behavioural and blood data was performed using the non-parametric sign test or, where applicable, the Fisher's exact test. These analyses were performed using Statistica v.10 (StatSoft). For the exploratory assessment of the relation between imaging and behavioural/blood measures, it was necessary to assess the association between imaging and clinical parameters, adjusting for the TSPO polymorphism. We did this by using multiple regression analysis. To be consistent in our use of non-parametric methods, we used a non-parametric regression method, Generalized Additive Models. To display the relationship between variables in scatter plots, we computed residuals adjusting for the effect of genotype. We performed these analyses using the *gam* library in the R statistics package (<http://www.R-project.org>). Statistical significance was determined by thresholding at $P = 0.05$.

Results

Higher brain TSPO levels in patients with chronic LBP

In the thalamic region of interest analysis, ^{11}C -PBR28 SUVs, normalized to whole brain, (SUVRs) were significantly higher in patients with chronic LBP than controls (left thalamus: $P \leq 0.01$; right thalamus: $P \leq 0.05$; Fig. 1A). The voxel-wise distribution of thalamic SUVRs (Fig. 1B) revealed that in control subjects non-zero

median voxel counts were observed only below values of 1.4, whereas in patients with chronic LBP a substantial number of voxels demonstrated values higher than 1.4 in both hemispheres (medians: 57.5 and 64 in the left and right thalamus, respectively).

The examination of individual thalamic SUVRs (Fig. 1C) shows that, strikingly, each patient exhibited higher SUVRs than his/her age-sex- and TSPO genotype-matched control in the thalamus. In all patients, the areas of maximal TSPO levels were consistently localized in dorsomedial subregions of the thalamus, as illustrated by the 3D rendering (Fig. 1C).

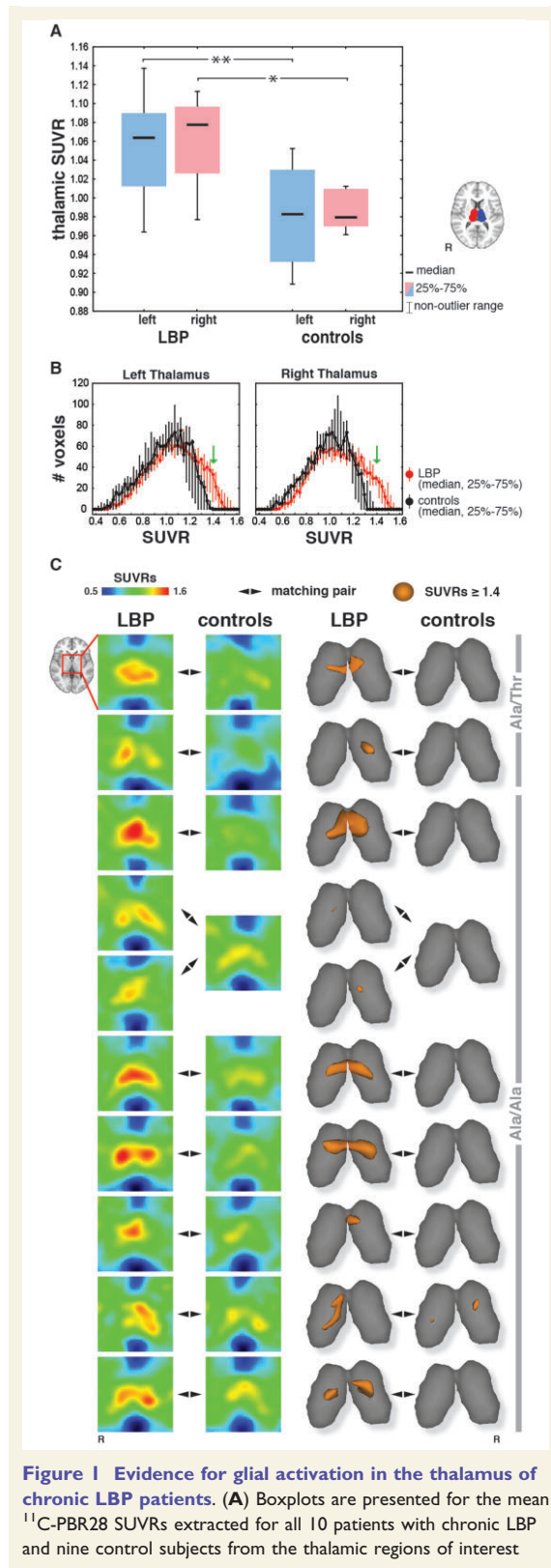
In the whole-brain voxel-wise analyses, SUVRs were significantly higher in thalamus, pre- and postcentral gyri and paracentral lobule (Fig. 2 and Supplementary Table 3). The peak group difference was observed in left thalamus, consistent with the mediodorsal nucleus. There were no brain regions for which the healthy controls showed statistically higher SUVRs than the patients with chronic LBP. When examined at the exploratory threshold of $P < 0.01$ uncorrected for multiple comparisons (Supplementary Fig. 1), additional regions demonstrated higher SUVRs in chronic LBP than controls, including insulae, middle cingulate cortex, ventromedial prefrontal cortex, posterior cingulate cortex, supplementary motor area and basal ganglia. Moreover, excluding the two patients on opioids (and their matching controls), yielded similar results (Supplementary Table 4). Thus, while exposure to opioids may also lead to a glial reaction (Watkins et al., 2009), our results do not seem to be confounded by opioid intake.

TSPO expression as a protective mechanism?

In the patients, ^{11}C -PBR28 imaging metrics, corrected for genotype, were negatively associated with pain outcome measures (Fig. 3) and circulating levels of proinflammatory cytokines (Fig. 3). First, the number of thalamic voxels with a SUVR value > 1.4 was negatively associated with pain levels at the time of scan, the total score on the McGill Pain Questionnaire ($P < 0.05$), and the levels of IL1B ($P < 0.05$), but not with IL6 or TNFA levels (P -values > 0.3). Second, the average SUVRs extracted from the thalamic cluster statistically significant in the voxel-wise analysis were negatively associated with levels of IL1B ($P < 0.001$) and IL6 ($P < 0.05$), but not with TNFA levels ($P = 0.38$). The association of thalamic ^{11}C -PBR28 SUVR with the McGill Pain Questionnaire total scores was negative, but did not reach statistical significance ($P = 0.11$). No association was observed between this imaging metric and pain levels during the scan ($P = 0.25$).

Discussion

Our study demonstrates the occurrence of glial activation, as measured by an increase in ^{11}C -PBR28 binding, in the brain of patients with chronic pain. Increased tracer



binding was observed most prominently in the thalamus, and with remarkable across-subject consistency (Fig. 1C).

Within the primary somatosensory and motor cortices (S1/M1) the SUVRs were higher in the putative sensorimotor representations of the lumbar spine [in the postcentral gyrus; Boendermaker *et al.* (2014), and leg (in the paracentral lobule); Loggia *et al.* (2012)]. This spatial pattern is consistent with the majority of patients suffering from pain in the lower back and leg(s), and is suggestive of somatotopically organized glial activation in S1/M1 (which in turn is also consistent with the observation that spinal glial activation generally follows somatotopic boundaries after unilateral spared nerve injury in rats) (Beggs and Salter, 2007).

In the last decade, animal research has led to the increased recognition of the importance of glial cells (such as microglia and astrocytes), and their interaction with neuronal cells, in the pathogenesis of pain conditions (Tsuda *et al.*, 2003; Watkins *et al.*, 2007; Calvo *et al.*, 2012; Ji *et al.*, 2013). For instance, nerve injury induces a profound activation and proliferation of spinal microglia (Liu *et al.*, 2000; Beggs and Salter, 2007; Echeverry *et al.*, 2008; Beggs *et al.*, 2012; Calvo and Bennett, 2012) and the upregulation of a variety of receptors in these cells, such as the adenosine triphosphate (ATP) receptor P2RX4 (also known as P2X₄) (Tsuda *et al.*, 2003) and the chemokine receptor CX3CR1 (Verge *et al.*, 2004), which induce hyperalgesia. Activated microglial cells produce inflammatory mediators, including proinflammatory cytokines and brain-derived neurotrophic factor (BDNF; Coull *et al.*, 2005; Ji and Suter, 2007) that activate or sensitize nociceptive neurons. The intrathecal injection of activated microglia produces tactile allodynia in naive rats, suggesting that microglial activation is sufficient to induce pain sensitization (Tsuda *et al.*, 2003). Finally, pharmacological inhibition of microglial activation prevents or delays neuropathic pain (Raghavendra *et al.*, 2003; Ledebor *et al.*, 2005). Similar to microglia, astrocytes have been shown to have a role in the induction and maintenance of pain sensitization (Ji *et al.*, 2006; Ren and Dubner, 2010; Ji *et al.*, 2013). For instance, in trigeminal models of inflammatory hyperalgesia (Guo *et al.*, 2007) or following trigeminal nerve injury (Okada-Ogawa *et al.*, 2009), astrocytes exhibit hypertrophy, and express enzymes, such as nitric oxide

Figure 1 Continued

(insert). The *P*-values refer to matched-pairs analyses (sign test) performed using nine chronic LBP-control matching pairs. The analyses were repeated twice, each time using one of the two patients matching the same control, with statistically significant results in both analyses. **P* < 0.05, ***P* < 0.01. (B) Voxel-wise distribution of thalamic SUVRs, showing that patients with chronic LBP have a substantial number of voxels at values ≥ 1.4 (green arrows), whereas controls have a median voxel count of 0. (C) Individual thalamic SUVRs are presented as axial sections (left), and 3D rendering of values higher than the threshold of 1.4 (right). Each row displays SUVRs for each patient-control matched pair. TSPO polymorphism (Ala/Ala or Ala/Thr) is indicated.

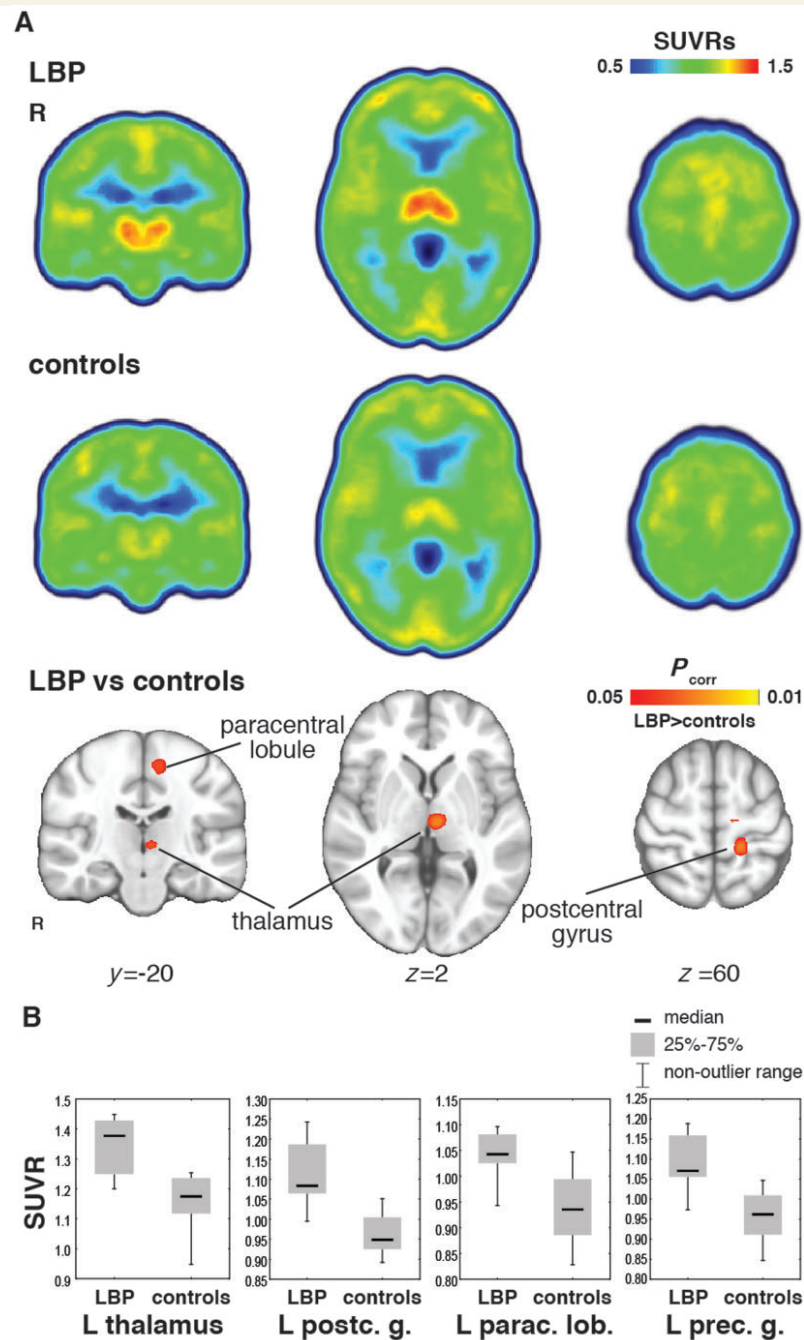


Figure 2 Whole-brain voxel-wise analyses. (A) Median SUVR map from healthy controls ($n = 9$) and patients with chronic LBP ($n = 10$) are presented. Matched-pairs tests (nine versus nine) revealed significantly higher TSPO levels in patients, in thalamus, pre- and postcentral gyri, and paracentral lobule ($P < 0.05$ corrected for multiple comparisons; permutation testing, 10 000 permutations). As two patients were matching the same controls, the analyses were performed first using the patient best matching the control. A second analysis was performed using the other patient, limiting our search to significant clusters from the first analysis, with identical results. (B) Boxplots for each of the four regions demonstrating statistically higher SUVRs in patients are shown for illustrative purposes. postc. = postcentral; g. = gyrus; parac. lob. = postcentral lobule; prec. = precentral.

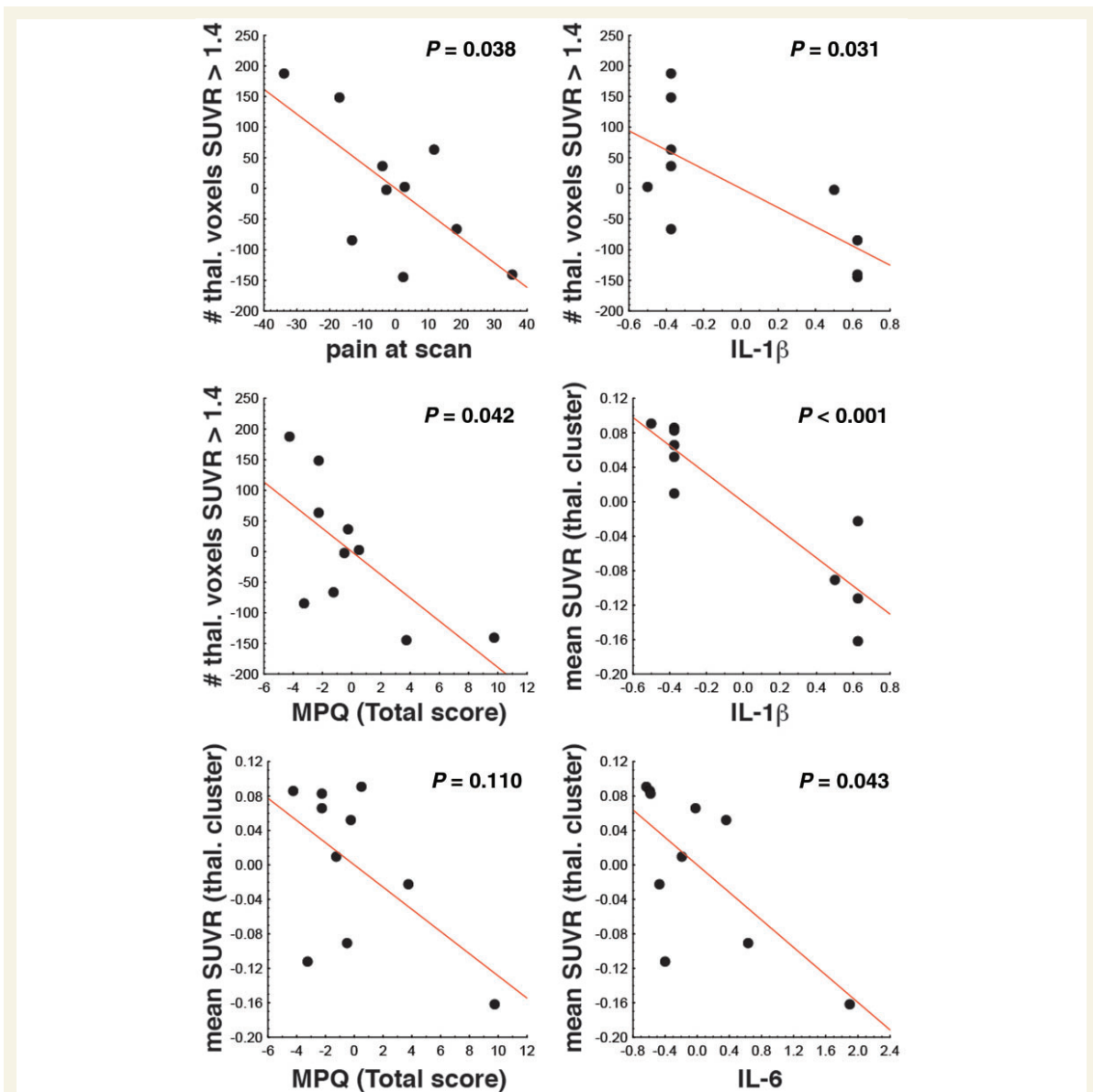


Figure 3 Anti-inflammatory and anti-nociceptive role of TSPO. SUVRs were negatively associated with pain outcomes (left) and blood levels of proinflammatory cytokines (right). The scatterplots show the residuals adjusting for the effect of genotype. MPQ = McGill Pain Questionnaire.

synthase (Meller *et al.*, 1994), as well as inflammatory mediators, such as the proinflammatory cytokine IL1B (Guo *et al.*, 2007) and chemokines such as CXCL2 (Chen *et al.*, 2014), that contribute to hyperalgesia and allodynia. Thermal and mechanical hyperalgesia are inhibited or attenuated by the injection of agents that disrupt either astroglial function (such as fluorocitrate, a glial metabolic inhibitor) (Meller *et al.*, 1994; Watkins *et al.*, 1997; Guo *et al.*, 2007; Okada-Ogawa *et al.*, 2009), or the action of glial products (such as IL1 receptor antagonists) (Watkins

et al., 1997). Taken together, these studies demonstrate that microglia and astrocytes play an important role in the pathogenesis of persistent pain in animals.

Although a plethora of animal studies has demonstrated that glial cells are involved in the establishment and maintenance of persistent pain, no study has previously demonstrated *in vivo* glial activation in humans suffering from chronic pain. Our observations have several potential implications. Firstly, they provide a rationale for exploring the role of glia as therapeutic target for chronic pain. In

animals, drugs that reduce glial activation (e.g. propentofylline and minocycline) have been found to potently inhibit proinflammatory cytokines, thereby suppressing the development of neuropathic pain (Mika, 2008; Leblanc et al., 2011). Importantly, some of these molecules are already FDA approved to treat human conditions of different aetiologies and testing for new, chronic pain-related indications would therefore be immediately possible. Two recent clinical trials (8- and 12-weeks long, respectively) have indicated that low-dose naltrexone (LDN) may have a clinically beneficial impact on fibromyalgia pain (Younger and Mackey, 2009; Younger et al., 2013). As LDN is thought to produce anti-inflammatory effects mainly by antagonizing the activity of glial cells (Mattioli et al., 2010), these studies suggest that glial modulators may be beneficial for fibromyalgia and perhaps other subgroups of chronic pain patients. On the other hand, it should be noted that some clinical trials have had negative results. A recent clinical trial assessing the efficacy of propentophylline to reduce pain in post-herpetic neuralgia was negative (Landry et al., 2012). Another study suggested that perioperative minocycline administration did not improve persistent pain after lumbar discectomy (Martinez et al., 2013). However, methodological concerns with these studies (Watkins et al., 2012) limit the significance of these negative study outcomes. In particular, the duration of trial design was unusually short in both studies. In the former study, propentophylline was administered for 4 weeks, and in the latter minocycline was administered peri-operatively for only 8 days, in both cases a significantly shorter duration than 12 weeks, as is more typically adopted in clinical trials. Moreover, as studies have shown that it is easier to prevent than reverse neuropathic pain using glial modulators (Raghavendra et al., 2003), longer trial durations may be needed to achieve therapeutic efficacy (Watkins et al., 2012). Other factors may also contribute to explain the negative results in these trials, including the dosage and potential interaction with other drugs or food intake (which may not have allowed for the drug to reach CNS sites at meaningful levels), or the choice of patient population (particularly for the propentofylline trial, as the pre-clinical evidence in support of a role for glia in post-herpetic neuralgia is more tenuous than in other chronic pain conditions) (Watkins et al., 2012).

The possibility to image pain-related glial activation *in vivo*, which we document in the present study, may help to identify patients most likely to benefit from this therapeutic approach, and to identify optimal treatment duration or dosage. Given the putative role of activated glia in many challenging issues associated with pain management, such as the induction of opioid-induced hyperalgesia and tolerance (Eidson and Murphy, 2013; Ferrini et al., 2013), the present findings offer clinical implications that may serve to guide future studies of the pathophysiology and management of a variety of persistent pain conditions.

In this study, glial activation was assessed using brain levels of TSPO, formerly called peripheral benzodiazepine

receptor (Banati et al., 1997; Papadopoulos et al., 2006). As experimental animal models and human post-mortem studies of CNS disorders have reliably shown concomitant and co-localized increases in TSPO expression and markers for activated astrocytes and/or microglia, TSPO expression is widely acknowledged as a marker of glial activation in CNS injury and disease. For instance, co-localization of glial activation and TSPO upregulation was observed in rodent models of experimental autoimmune encephalomyelitis, in rodent models of multiple sclerosis, as well as in human multiple sclerosis lesions (Vowinckel et al., 1997; Banati et al., 2000; Chen et al., 2004; Chen and Guilarte, 2006; Ji et al., 2008; Cosenza-Nashat et al., 2009; Abourbeh et al., 2012), in non-human primate models of HIV encephalitis (Venneti et al., 2007; Cosenza-Nashat et al., 2009), as well as in human HIV encephalitis (Cosenza-Nashat et al., 2009), in both human post-mortem and experimental rodent models of ischaemia (Rojas et al., 2007; Cosenza-Nashat et al., 2009; Martin et al., 2010) and Alzheimer's disease (Ji et al., 2008; Cosenza-Nashat et al., 2009; Gulyas et al., 2009), and in rodent models of ethanol and trimethyl neurotoxicity (Kuhlmann and Guilarte, 2000; Maeda et al., 2007). More pertinent to our study, TSPO was upregulated in spinal astrocytes and microglia in Complete Freund's Adjuvant (CFA)-induced monoarthritis of the tibio-tarsal joint (Hernstadt et al., 2009) and following L5 spinal nerve ligation pain (Wei et al., 2013). Given these observations, the increased ^{11}C -PBR28 levels we observed in patients with chronic pain can be interpreted as evidence of glial activation. Interestingly, the involvement of glial subtypes in neuroinflammatory responses seems to depend on the time course of disease. In several animal models, initial TSPO upregulation following acute CNS insult is accompanied by a predominantly microglial response that typically peaks and begins dissipating several days to weeks after injury. This rapid microglial response is paralleled by a delayed but steadily increasing astrocytic component (Kuhlmann and Guilarte, 2000; Chen et al., 2004; Chen and Guilarte, 2006; Martin et al., 2010; Liu et al., 2014). Human post-mortem data seem to corroborate this phase-dependent glial contribution: in acute multiple sclerosis lesions, microglia and macrophages represent most TSPO+ cells, whereas astrocytes are the dominant TSPO+ cells in chronic, silent lesions (Cosenza-Nashat et al., 2009). Because our patients suffered from years of pain, it is plausible that astrocytes provided a significant contribution to the increased PET signal observed in our data.

Although TSPO is a marker of activated glia, a phenomenon thought to be responsible for the amplification of pain signals within the CNS (Ji et al., 2013), TSPO expression itself has been shown in animals to exert inhibitory effects on neuroinflammation (Wei et al., 2013; Bae et al., 2014; Wang et al., 2014), and to promote recovery from neuropathic pain (Wei et al., 2013), likely through the stimulation of steroidogenesis (Batarseh and Papadopoulos, 2010; Wei et al., 2013). In fact, studies suggest that one of the

functions of TSPO in activated glia is to limit the magnitude of inflammatory responses after their initiation (Wang *et al.*, 2014). Our observation that ^{11}C -PBR28 SUVRs negatively correlate with levels of circulating proinflammatory cytokines and pain further corroborate the hypothesis that TSPO expression has anti-inflammatory and pain-protective effects. The negative correlations found in our study therefore support the contention that TSPO ligands may be a novel therapeutic target for the treatment of pathological pain (Wei *et al.*, 2013), as previously suggested for a variety of conditions (Rupprecht *et al.*, 2009, 2010). As TSPO expression is upregulated in activated glia and was found to negatively correlate with peripheral cytokines and pain in our study, low levels of TSPO might be interpreted differently between groups. For example, in healthy volunteers, low levels could simply reflect low levels of glial activation. On the other hand, patients with chronic pain exhibiting lower TSPO levels may have impairments in TSPO expression in activated glia, and therefore in their ability to limit the glial responses after their initiation. Clearly, additional studies are required to elucidate the relationship between ^{11}C -PBR28 and peripheral markers of inflammation, particularly as peripheral cytokine levels are often found to be decoupled from those within the CNS (Bromander *et al.*, 2012).

Future studies, including some currently already underway in our laboratory, will need to determine whether different pain populations present differences in the spatial distribution of glial activation. The discovery of 'glial signatures' of chronic pain states might lead to the identification of objective imaging markers that could (i) complement the patient's subjective assessment and other measures (e.g. quantitative sensory testing) to guide clinical practice; and (ii) reduce the patient heterogeneity which has traditionally led to poor signal-to-noise ratios in most clinical drug evaluation studies (Gomez-Mancilla *et al.*, 2005). Finally, as glial cells respond to very subtle changes in their microenvironment that even precede pathological changes that are detectable histologically (de Vries *et al.*, 2006; Cagnin *et al.*, 2007), glial activation might be an early marker of the alterations that have been shown to occur in the brains of chronic pain patients (Tracey and Bushnell, 2009). This might allow early identification of individuals at risk of transitioning from acute to chronic pain, thus optimizing treatment strategies.

In sum, our findings demonstrate a role of glia in human pain disorders, support the role of the assessment of glial activation and TSPO expression in selective brain areas as an imaging marker and potential treatment target for chronic pain disorders in humans.

Acknowledgements

We would like to thank Patricia McCarthy, Nancy Shearer and the Massachusetts General Hospital Clinical Research Centre for providing nursing support, Drs Douglas Greve,

Marc Normandin, Nicolas Guehl and Jean Logan for help with data processing, Dr Julie Price for helpful comments, Dr Nazem Atassi for sharing imaging resources, Drs Randy Gollub and Alexander Guimaraes for their help with obtaining informed consent, Ms Jiaxuan (Jessie) Wang for her support with the study logistics and Ms Ekaterina Protsenko for editorial assistance.

Funding

This project was supported by Harvard Catalyst Advanced Imaging Pilot Grant (J.M.H.), 1R21NS087472-01A1 (M.L.L.), 1UL1TR001102-01, 8UL1TR000170-05, from the National Centre for Advancing Translational Science, and 1UL1RR025758-04 from the National Centre for Research Resources, Harvard Clinical and Translational Science Centre, and financial contributions from Harvard University and its affiliated academic healthcare centres. The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic healthcare centres, or the National Institutes of Health. The authors have no conflicts of interest or competing financial interests to declare.

Supplementary material

Supplementary material is available at *Brain* online.

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